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Bioactive steroids from *Oryza sativa* L.

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ABSTRACT

Rice is one of the most interesting crops in the world from both the social and the economic point of views. The monoculture practices along with the heavy use of herbicides are characteristic of modern agriculture and are inducing the appearance of tolerant and/or herbicide resistant weed biotypes. This is the case the world's main weed of rice barnyardgrass (*Echinochloa crus-galli*). Alternative strategies for weed suppression consist of the use of chemicals from rice due to necessity of obtaining new herbicides with new modes of action that could prevent resistance phenomena.

In order to carry out a study that guides to the isolation of the most active compounds from rice, different extracts were achieved, and their activities evaluated. So, all the plant material was divided into three parts: fresh plant, dried plant, and fresh plant from Pluviotron. The aerial part was separated from roots in all cases and extracted in water, in organic solvents as well as with the Pluviotron device.

The activity of the 12 extracts obtained was evaluated using a generalist bioassay, wheat etiolated coleoptiles bioassay, and a phytotoxic bioassay on barnyardgrass as target species. The bioactive extracts were fractionated and 15 compounds were isolated and identified by spectroscopic methods. Eight of these compounds were isolated for the first time in *Oryza sativa*.

The most phytotoxic compounds on *E. crus-galli* were ergosterol peroxide and 7-oxo-stigmasterol. In the case of ergosterol peroxide the activity was higher than the commercial herbicide Logran. This is the first report of potential allelopathic activity of steroids on weeds based on their phytotoxicity.

1. Introduction

Rice (*Oryza sativa* L.) is the most important food crop in the world. Herbicides and fungicides are heavily used for optimum yields and maximum profit, which may cause environmental problems in paddy ecosystems [1,2]. Maintaining an adequate flood and, in some cases, intensive hand labor is important to managing weeds in rice. Combined with improved cultural and fertility practices and the development of high yielding varieties, selective herbicides have dramatically increased rice yields in the last 50 years [3]. Nevertheless, an adequate sustainable agricultural practice requires new strategies to improve weed and pathogen management.

Allelopathy offers a new approach for the discovery of new lead compounds and their use as herbicides and pesticides from plants, fungi, and microorganisms. The term "Allelopathy" has undergone several changes over time [4–6]. The definition adopted by the International Allelopathy Society (IAS) in 1996 is "The science that studies any process involving secondary metabolites produced by plants, algae, bacteria, and fungi that influences the growth and development of agricultural and biological systems". Allelopathic

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interactions derive from the production of secondary metabolites for defense by plant and microorganisms. The secondary metabolites involved are called allelochemicals [7]. These allelochemicals are bioactive natural products that induce a wide array of biological effects and their understanding could provide great benefits on agriculture and weed management.

Aqueous extracts of rice plants [8] and their decomposing residues [9] inhibit the growth of several species. Extensive allelopathic studies in rice plants have shown a wide variety of compounds, mainly phenolics [1,10,11] and some diterpenes [12]. However, phenolic acids are unlike to explain the allelopathy in rice since their soil concentrations never reach phytotoxic levels [13]. In this paper, we report the isolation of eight steroids from rice active extracts, five of them for the first time from *O. sativa*, and their biological activities being described. The most active compound was ergosterol endoperoxide (**7**). This compound has been reported as an example of steroid with the necessary structural requirements for effectiveness against *Mycobacterium tuberculosis* including a pedant polar head group, a flexible hydrophilic chain, a long and rigid hydrophobic unit and a double bond in the side-chain [14]. Other activities reported for **7** are inhibitory on Gram-negative and -positive bacteria, such as *Pseudomones* spp. and *Bacillus subtilis* [15]; immunosuppressive, antiviral, anti-inflammatory, and antitumor [16–19]. This is the first report of potential allelopathic activities of steroids on weeds based on their phytotoxicity.

Bioactivity has been tested using two different bioassays: etiolated wheat coleoptiles and phytotoxicity. The first one provides an initial overview of the bioactivity. The target species for evaluation of the phytotoxicity was *Echinochloa crus-galli* (L.) Beauv. (barnyardgrass). This is considered one of the most problematic weeds, especially in rice crops [20]. Barnyardgrass is widely distributed, and constitutes a serious weed problem in 42 countries and has been found, at least, in 27 more [21]. It is the world's main weed of rice affecting nearly 36 crops worldwide. It reduces rice tillering by 50%, and also reduces the number of panicles, height, weight of grains, and number of grains per panicle; rice yields may be reduced by 2000–4000 kg ha^{-1} [20]. Barnyardgrass has been proven to reduce the yields of potatoes [22], snap beans [23], corn [24], grain sorghum [25], sugarbeets, green peas, and melons [26]. Barnyardgrass is also a host to many viruses of rice and other grass crops [20]. It is also a host for *Striga asiatica*, which infests sorghum, corn, millet, sugar-cane, rice, and tobacco in India, Africa, and the United States [27].

2. Experimental

2.1. General

IR spectra (KBr) were recorded on a Perkin-Elmer FT-IR Spectrum 1000, Matton 5020 spectrophotometer. NMR spectra were run on Varian INOVA-400 and Varian INOVA-600 spectrometers. Chemical shifts are given in ppm with respect to residual $CHCl_3$ or $CDCl_3$ signals (δ, 7.25 and 77.00, respectively). FAB-MS and HRMS were carried out on VG 1250 and VG AUTOESPEC mass spectrometers (70 eV).

2.2. Plant material

The fresh plant of *O. sativa* cv. Puntal was collected in Isla Menor (Seville) in June 2002 at the stage next to harvest. Plant material was provided by the company Dow AgroScience.

2.3. Extraction and isolation

The plant material was divided into three parts, i.e. fresh plant, dried plant, and fresh plant for Pluviotron. The aerial plant was separated from roots in all cases.

Fresh aerial part (3 kg) and fresh roots (1.5 kg) of *O. sativa* cv. Puntal were extracted in water for 24 h at room temperature in the dark to yield 52.5 and 14.7 g of the crude extracts named FAPW and FRW, respectively. The plant residues were dried at room temperature and extracted with CH_2Cl_2 (48 h) and later with methanol (48 h) yielding after solvent removal the following extracts: 6.9 g from fresh aerial part in DCM (FAPDCM), 6.9 g from fresh aerial part in methanol (FAPMe), 1.6 g from fresh roots in DCM (FRDCM), and 4.2 g from fresh roots in methanol (FRMe).

Dried aerial parts (500 g) and dried roots (200 g) were extracted in DCM and later in methanol for 48 h at room temperature yielding after solvent removal 6.2 g of crude extract in DCM from dried aerial part (DAPDCM), 33.5 g of crude extract in methanol from dried aerial part (DAPMe), 1.3 g of crude extract in DCM from dried roots (DRDCM), and 8.7 g of crude extract in methanol from dried roots (DRMe).

One kilogram of aerial parts and 450 g roots of fresh plant were extracted in water using the rain simulator device Pluviotron (24 h) to yield 1.2 g of the crude extract APP, and 0.4 g of the crude extract RP, respectively. [28]

The 12 extracts were bioassayed on wheat etiolated coleoptiles and barnyardgrass (*E. crus-galli*). Extracts in DCM from dried aerial part (DAPDCM) and from fresh roots (FRDCM) were the most active. These extracts were chromatographed on silica gel using hexane–EtOAc mixtures of increasing polarity as eluant. Fractions eluted between hexane–EtOAc 7:3 and 5:5 yielded compounds **1** (200 mg), **2** (1.7 mg), **3** (300 mg), **4** (19 mg), **5** (3.5 mg), **6** (19 mg), **7** (12 mg), and **8** (1 mg) (Fig. 1).

2.4. Biological activity

2.4.1. Coleoptile wheat bioassay

Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in 15 cm diameter Petri dishes moistened with water and grown in the dark at 22 ± 1 °C for 3 days [29]. The roots and caryopsis were removed from the shoots, that were placed in a Van der Weij guillotine and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for bioassay. All manipulations were performed under a green safelight [30]. Crude extracts solutions were prepared in a phosphate–citrate buffer solution containing 2% sucrose [30] at pH 5.6 at 600, 300, and 150 ppm. Parallel controls were also run.

Solutions (2 mL) were added to each test tube. Following the placement of five coleoptiles in each test tube (three tubes per dilution), the tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at 22 °C in the dark. The coleoptiles were measured by applying digital photography. The assay was run

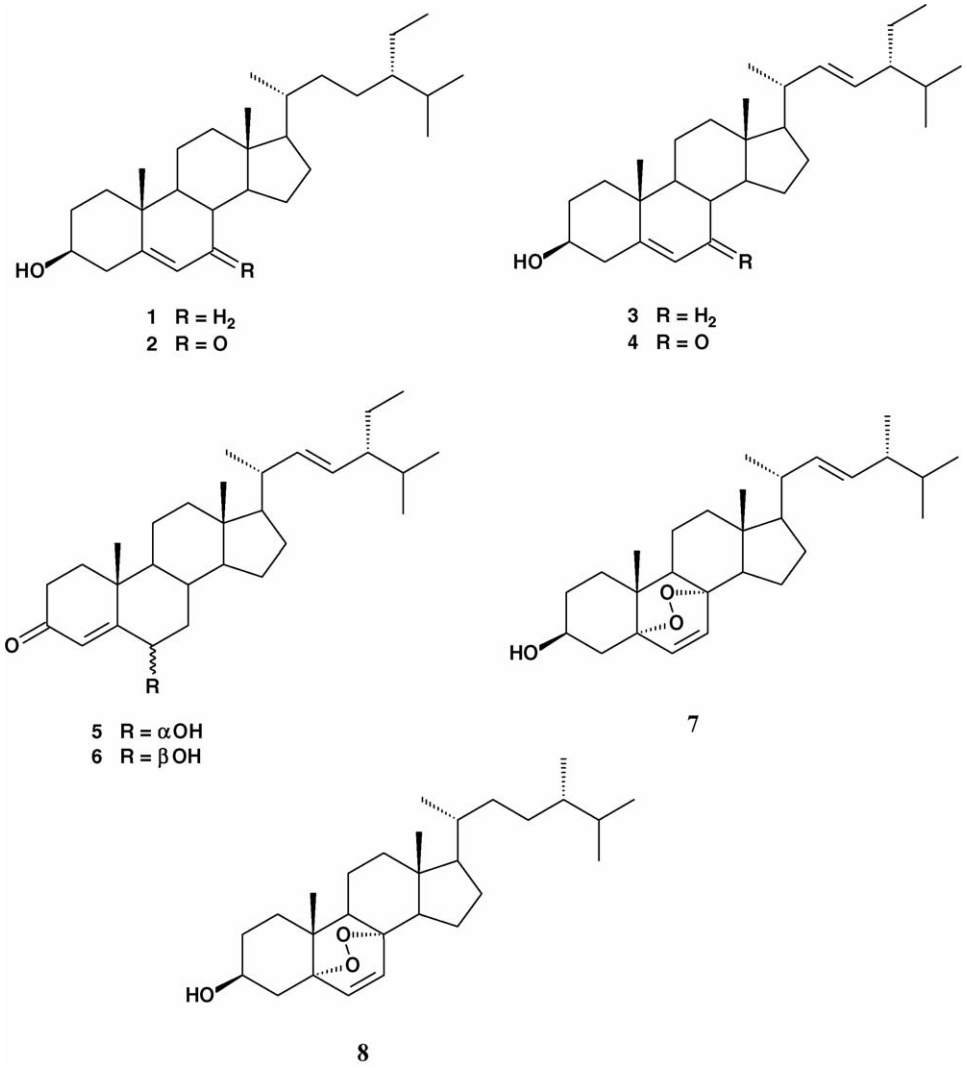

Fig. 1 – Compounds isolated from active fractions of rice extracts.

in duplicate. Data were statistically analyzed using the Welch's test [31] and presented as percentage differences from control. Thus, zero represents the control; positive values represent stimulation of the studied parameter; and negative values represent inhibition.

2.4.2. Phytotoxicity bioassay

2.4.2.1. *Methodology.* Bioassays used Petri dishes (90 mm. diameter) fitted with one sheet of Whatman no.1 filter paper as support. Germination and growth were conducted in aqueous solutions at controlled pH by using 10^{-2} M 2-[*N*-morpholino]ethanesulphonic acid (MES) and addition of solution of NaOH 1 M (pH 6.0). Compounds to be assayed were dissolved in DMSO (0.2, 0.1, 0.02, 0.01, and 0.002 M), and these solutions were diluted with buffer (5 μL DMSO solution/mL buffer) to reach test concentrations (1 mM, 500, 100, 50, and 10 μM).

E. crus-galli seeds were purchased from Herbiseed Co. (Twyford, England) and used as received. The number of seeds in each Petri dish was 25. 5 mL of treatment, control or internal reference solution were added to each Petri dish. Four replicates were used (100 seeds).

After adding seeds and aqueous solutions, Petri dishes were sealed with Parafilm to ensure closed-system models. Seeds were further incubated at 25 °C in a Memmert ICE 700 controlled environment growth chamber, in absence of light. The bioassay was run over a 5 days time period. Finally, plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process. This helped the handling of the plants and allowed a more accurate measurement of root and shoot lengths.

Commercial herbicide Logran©, a combination of *N*-(1,1-dimethylethyl)-*N*-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (Terbutryn, 59.4%) and 2-(2-chloroethoxy)-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide (Triasulfuron, 0.6%) was used as internal reference, according to a comparison study previously reported [32]. It was used at the same concentrations (1 mM, 500, 100, 50, and 10 μM) and in the same conditions that the compounds in study. Buffered aqueous solutions with DMSO

and without any tested compound were used as control samples.

2.4.2.2. *Bioassay data acquisition*. Germination rate, root length and shoot length were recorded by using a Fitomed © system [33], that allowed automatic data acquisition and statistical analysis by its associated software.

2.4.2.3. *Statistical analysis*. Data were statistically analyzed using the Welch's test, with significance fixed at 0.01 and 0.05 and presented as percentage differences from control in the same way as for coleoptiles. [32,33].

3. Results and discussion

Plant material was splitted into fresh and dried plants. Also aerial parts were separated from roots, and each one extracted with water, organic solvents, and the Pluviotron device. Soft extraction conditions simulating natural rain were used in the Pluviotron extractor, whereas organic solvents (DCM and methanol) were also used for soaking plant material during 48 h.

The activity of the twelve extracts obtained was evaluated on etiolated wheat coleoptiles (Fig. 2) and on *E. crus-galli* (Fig. 3).

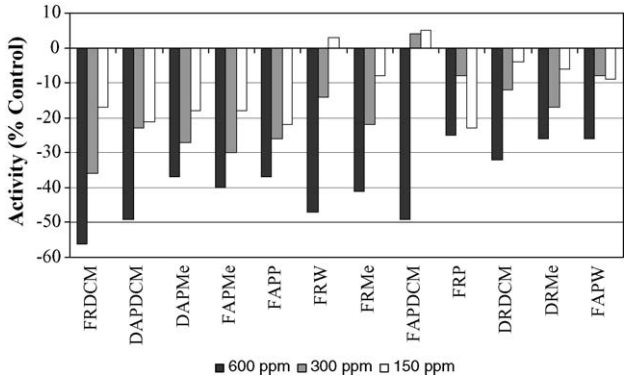

Fig. 2 – Effects of rice extracts (dry extract weight/water, w/v) on wheat coleoptiles elongation.

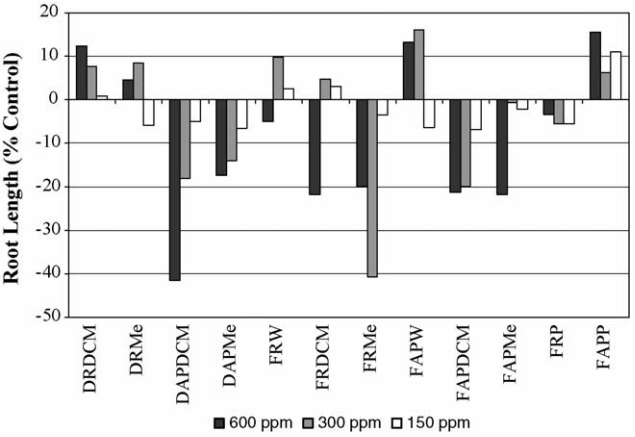

Fig. 3 – Effects of rice extracts (dry extract weight/water, w/v) on the weed *Echinochloa crus-galli*.

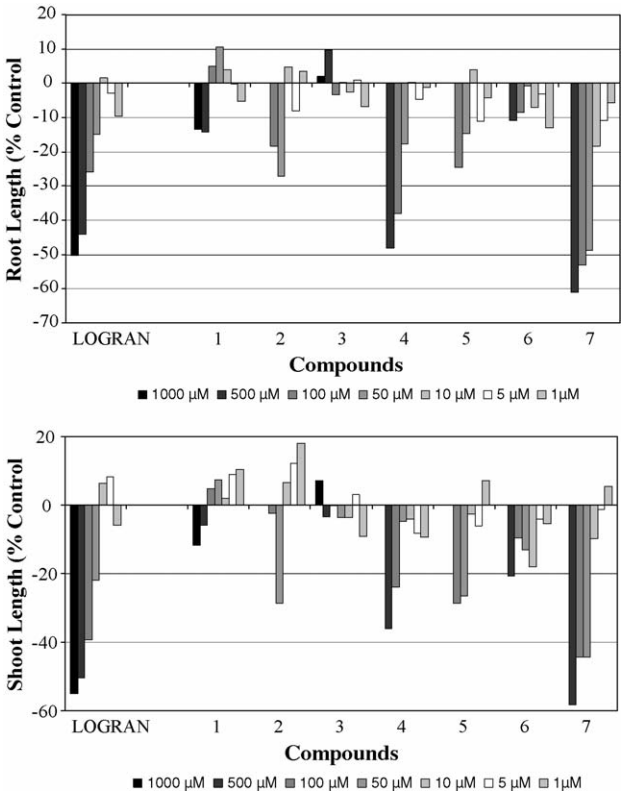

Fig. 4 – Effects of compounds 1–7 on the growth of *Echinochloa crus-galli*.

The results of bioassay on etiolated wheat coleoptiles showed that extracts dried aerial part in DCM (DAPDCM) and fresh root in DCM (FRDCM) present the highest levels of activity with values near to −60 and −50% of inhibition at 600 ppm, respectively. DAPDCM inhibited coleoptiles growth by −36% at 300 ppm. These results were confirmed with the second bioassay of phytotoxicity on *E. crus-galli*. Both extracts showed the best values of growth inhibition for this weed, close to those of fresh roots in methanol (FRMe) and fresh aerial parts in methanol (FAPMe).

Extracts from dried aerial parts in DCM (DAPDCM) and from fresh roots in DCM (FRDCM) were the most active extracts in both bioassays and were selected for chemical study. Chromatography using hexane-EtOAc 7:3 and 5:5 as eluent yielded compounds **1–8** (Fig. 1). The spectroscopic data of these compounds were identical to those previously reported for β-sitosterol (**1**) [34], 7-oxositosterol (**2**) [34], stigmasterol (**3**) [35], 7-oxostigmasterol (**4**) [36], (6α,22*E*)-hydroxy-stigmata-4,22-dien-3-one (**5**) [37], (6β,22*E*)-hydroxy-stigmata-4,22-dien-3-one (**6**) [38], ergosterol peroxide (**7**) [39] and 5α,8α-epidioxy-24(*R*)-methylcolesta-6-en-3β-ol (**8**) [39]. Compounds **2**, **4–6,** and **8** have not been isolated from *O. sativa* previously.

Seven out of the eight steroids isolated from *O. sativa* cv. Puntal were subjected to bioassays. Unfortunately, not enough amounts of 5α,8α-epidioxy-24(*R*)-methylcolesta-6-en-3β-ol (**8**) were available for the bioassay, The amounts of isolated compounds allowed us to assay compounds **1** and **3** from 1 mM; **4**, **6**, and **7** from 500 μM; and, finally, **2** and **5** from 100 μM (Fig. 4).

The results showed that 7-oxo-stigmasterol (**4**) and ergosterol peroxide (**7**) are compounds with the highest inhibition values, around −50% (500 μM) for **4** on the root length and the −60% (500 μM) for **7** in shoot and root lengths. Significant values of shoot growth inhibition were obtained for **5** at the highest concentrations tested (−29 and −27% at 100 and 50 μM, respectively). Compound **7** showed higher inhibition than the commercial herbicide Logran especially at lower concentrations: −50% of growth inhibition at 50 μM in comparison with Logran (−25% at 50 μM).

Some structure–activity relationships can be inferred from the phytotoxicity bioassay. Compounds **4** and **7** showed the most significant values of phytotoxicity. However, they present different functionalization pattern in the B-ring. The comparison with **3**, which is not active, suggests that a higher oxidation level in ring B enhances the phytotoxic activity on *E. crus-galli*. Activity can also be associated with the presence of a double bond in the side-chain, thus, **4** is clearly more active than **2**.

Regarding to the functional groups present in the active molecules, There is an α,β-unsaturated carbonyl moiety in **4** and an endoperoxide group in compound **7**; the first one can act as Michael acceptor with nucleophillic residues located in biomolecules.

On the other hand, endoperoxides can be transformed by peroxydases, very common in vegetable tissues. Moreover, endoperoxides could provoke the appearance of free radicals that could be responsible of cellular damages.

These results, suggest that steroids may be involved in the defense mechanism of rice, pointing out the importance of compounds **4** and **7**. Nevertheless, to establish the specific ecological roles of these compounds some field experiments should be carried out.

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